

***Cryptosporidium* Pig Genotype II Diagnosed in Pigs From the State of Rio De Janeiro, Brazil**

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ABSTRACT: Pigs may represent a source of *Cryptosporidium* sp. infection to humans. The objective of this study was to identify the *Cryptosporidium* species present in pigs from the State of Rio de Janeiro, Brazil, and verify what risks pigs represent in the transmission of human cryptosporidiosis, because there is no such information to date in Brazil. Ninety-one samples of pig feces were collected from 10 piggeries in 2 municipalities located in the north and northwest regions of the State of Rio de Janeiro, Brazil. A nested polymerase chain reaction (PCR) protocol to amplify an 830-bp fragment of the small subunit rDNA (SSU rRNA) gene was followed by sequencing of all positive PCR samples. Two samples (2.2%) were *Cryptosporidium* sp. positive and were identified as pig genotype type II (PGII). This genotype has been observed in an immunocompetent person, in cattle without pigs nearby, and from a potential human source. Its potential for zoonotic transmission is little known and should be rigorously studied.

Brazil is the fourth largest producer and exporter of pork worldwide (ABIPECS, 2009), with a national herd estimated at 38 million animals (IBGE, 2009). The main industrial production is located in the south and southeastern regions, where high production levels have been obtained through advances in genetics, nutrition, reproduction, handling, and sanitation (Nishi et al., 2000). Production in an intensive system may cause proliferation and maintenance of pathogenic agents if techniques of handling are inadequate (Calderaro et al., 2001). Intestinal parasites are among the pathogens causing diarrhea and loss of development, especially in piglets, resulting in a loss of productivity (Nishi et al., 2000).

Pig cryptosporidiosis is generally asymptomatic, but may also cause significant economic loss (Bilic and Bilkei, 2006) and can be a source of infection to humans and other animals that have direct or indirect contact with pig manure (Xiao et al., 2002). Cryptosporidiosis can thus be opportunistic. Immunosuppression resulting from other pathogens, such as infection with Porcine Circovirus Type 2, can exacerbate *Cryptosporidium* sp. infection (Núñez et al., 2003).

In spite of Brazil being one of the largest producers of pork, there are still herds with poor sanitary conditions. In the State of Rio de Janeiro, Brazil, almost all pig production facilities that were examined had poor hygienic conditions and were situated near private homes and rivers (Fiuza et al., 2008). *Cryptosporidium* sp. oocysts were identified by microscopy at these locations (Fiuza et al. 2008). However, there is no information about *Cryptosporidium* species present in Brazilian pigs. Consequently, it is not known what risks pigs represent for transmission of cryptosporidiosis to humans. The present study was designed to identify *Cryptosporidium* species in pig feces using molecular techniques.

Ninety-one samples of pig feces (15 g each) were randomly obtained from 10 piggeries situated near homes in the municipalities of Campos dos Goytacazes and Itaperuna, located in the north and northwest regions of the State of Rio de Janeiro, respectively. Samples from 10% of animals in each piggery were collected directly from the floor, immediately after defecation, with care to collect only the portion that did not have direct contact with the pavement. Feces were collected from pigs approximately 1- to 12-mo-old. For each sample, individual plastic bags were labeled and placed in isothermal boxes (8–10 C) and then immediately transported to the Veterinary Hospital of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF) at the Medical Clinic of the Laboratório de Sanidade Animal (LSA) that is in the Centro de Ciências e Tecnologias Agropecuárias (CCTA).

The samples were processed by centrifugation with sucrose (1.1 g/ml) to concentrate and purify oocysts according to Fiuza et al. (2008). The sugar concentration method used by Fiuza et al. (2008), followed by the nested PCR used in this study, has a diagnostic sensitivity of 10, 40, and 80% in samples previously spiked with 10, 100, and 1,000 *Cryptosporidium parvum* oocysts per g of feces, respectively.

For DNA extraction, the DNeasy Tissue Kit (Qiagen, Valencia, California) was used with reagents provided by the manufacturer. Modifications of the protocol included overnight incubation with proteinase K and elution in 100 µl of AE buffer to increase the quantity of recovered DNA (Santín et al., 2004).

The polymerase chain reaction (PCR) and sequencing procedures were performed at the Environmental Microbial and Food Safety Laboratory (EMFSL) at the United States Department of Agriculture (USDA), Beltsville, Maryland.

A nested PCR protocol was used to amplify an 830-bp fragment of the SSU rRNA gene according to Santín et al. (2004). Before sequencing, the PCR product was purified with 2 hydrolytic enzymes: Exonuclease I and Shrimp alkaline phosphatase in a specially formulated buffer (ExoSAP-IT, USB Corporation, Cleveland, Ohio). After purification, the product was sequenced in both directions using the same PCR primers in 10-µl reactions, with Big Dye Chemistries, in an ABI 3100 sequencer analyzer (Applied Biosystems, Foster City, California). The sequences of each strand were aligned and examined with Lasergene software (DNASTAR, Madison, Wisconsin). The obtained sequences were submitted to the Basic Local Alignment Search Tool (BLAST) analysis to identify similarities with the GenBank sequences (Altschul et al., 1997).

Samples (2.2%) from 2 pigs from Itaperuna were *Cryptosporidium* sp.-positive by PCR. They were identified as pig genotype II (PGII) by sequence analysis (Genbank EU331243 and DQ182600). GenBank accession numbers assigned to the nucleotide sequences determined in this study are GQ924104 and GQ924105.

To the best of our knowledge, there is no scientific literature on the prevalence of *Cryptosporidium* sp. in pigs in Brazil based on molecular techniques. Using microscopy, Nishi et al. (2000) identified intestinal parasites in pigs in the States of Minas Gerais (MG) and São Paulo (SP). *Cryptosporidium* sp. oocysts were identified in 1.7 and 7.6% of pigs in MG and SP, respectively. In another survey conducted in SP, only 2 (1.2%) of 174 pre-weaned piglets presenting with diarrhea had *Cryptosporidium* sp. oocysts in their feces. Because other pathogenic agents were found in these feces, diarrhea could not be attributed to cryptosporidiosis (Calderaro et al., 2001). In diarrheic feces from piglets, 2.1% contained *Cryptosporidium* sp. oocysts (Martins et al., 1993). In another study in SP, 77 (10.27%) of 750 samples from piglets contained *Cryptosporidium* sp. oocysts (Coutinho et al., 2003).

Recently, 32% of pigs on farms in the State of Rio de Janeiro were found positive for oocysts by microscopy (Fiuza et al., 2008). In contrast, only 2.2% of pigs on farms in the State of Rio de Janeiro were positive in the present study using molecular methods. According to Morgan et al. (1998), false-positive results may occur when the diagnosis is performed only by microscopy, without molecular confirmation. Possibly, the collection of only 1 sample per animal in the present study might have contributed to the low prevalence, because some animals could have been excreting low daily numbers of oocysts or excreting them intermittently.

Reports of cryptosporidiosis in pigs have described a wide range of prevalence in many countries, based on microscopic and molecular techniques. Prevalence has ranged from 1.4 to 100% (Atwill et al., 1997; Wieler et al., 2001; Guselle et al., 2003; Ryan et al., 2003; Yu and Seo, 2004; Zintl et al., 2007; Johnson et al., 2008). Post-weaned pigs (>4 wk of age) were most frequently affected (Maddox-Hyttel et al., 2006; Vitovec et al., 2006).

Among the species and genotypes reported in pigs, *Cryptosporidium suis* and PGII were the most prevalent (Johnson et al., 2008). Studies show a relationship between the age of the animal and the *Cryptosporidium* species detected. Thus, *C. suis* was usually found in pre-weaned pigs (younger than 4 wk of age) while PGII was typically found in post-weaned pigs (Ryan et al., 2005; Zintl et al., 2007; Johnson et al., 2008). Similar to pigs in Brazil, in the present study, 2.9% of intensively farmed 4-wk- to 6-mo-old pigs in Ireland were parasitized with PGII (Zintl et al., 2007). Furthermore, the sequence of nucleotides found in Ireland was identical to the sequence found in the present study (GenBank DQ182600).

Other studies around the world have detected a high prevalence of PGII in different production systems and ages, i.e., 11.1% in Australia (Johnson et al., 2008), 11.2% in Spain (Suárez-Luengas et al., 2007), 25% in the Czech Republic (Kvác, Hanzlíková et al., 2009), and 13.8 and 36.2 in Denmark (Langkjær et al., 2007). Except for the study in Australia, the studies cited above may underestimate the real prevalence of this genotype because molecular studies have been performed only on samples positive by microscopy. Molecular techniques have a greater power of diagnosis (Ryan et al., 2005). Therefore, its use is recommended in all samples for the most accurate estimate of prevalence data of *Cryptosporidium* species in a particular region.

The PGII nucleotide sequence found in this study (GQ924104) was recently reported in a 29-yr-old immunocompetent man (GenBank EU331243) as being the first diagnosis of this genotype in humans (Kvác, Kvitová et al., 2009).

Despite this first report in humans, the presence of only PGII still does not appear to be a public health risk because it has been reported almost entirely in pigs. However, Langkjær et al., (2007) observed this genotype in a calf from a property in Denmark without any pigs nearby. This fact, combined with the detection of this genotype in water catchment areas (Ryan et al., 2005), should be treated with caution due to the possibility of transmission and spread of this genotype to other farms and recreational waters (Langkjær et al., 2007). Nevertheless, according to Ryan et al. (2005), the potential for zoonotic transmission of this genotype should be thoroughly examined because it was detected from a potential human source.

The confirmation of PGII EU331243 and DQ182600 in geographically distant countries suggests a cosmopolitan distribution of this genotype.

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